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Characterization of a Gelatinase from Human Rheumatoid Synovial Fluid Cells

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Summary: A metalloproteinase with a specificity for gelatin was isolated from serum-free medium of cultures of rheumatoid synovial fluid. The enzyme showed all the properties of a leukocyte gelatinase. In addition to gelatin this proteinase cleaved the synthetic substrate dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-*D*-Arg (Dnp-peptide) rapidly, while casein was a much poorer substrate. This proteinase showed no enzymatic activity against collagen type I, was secreted in a latent form and could be activated by trypsin or organomercurial compounds, such as mersalylic acid or 4-aminophenylmercury acetate. The latent enzyme had an apparent molecular mass of 130 000–150 000 estimated by gel filtration or 97 000 by electrophoresis on polyacrylamide gel containing sodium dodecyl sulphate. When analysed by immunoblotting the enzyme was recognized by antibodies raised against human polymorphonuclear leukocyte gelatinase. Although we found synovial fibroblasts to be largely present in the cell cultures we could not detect any fibroblast gelatinase activity.

Introduction

Three related neutral metalloproteinases – interstitial collagenase, gelatinase and stromelysin – are thought to play the major role in the degradation of the extracellular matrix compounds, collagen and proteoglycan. The classic vertebrate collagenases, now widely called interstitial collagenases (1), exhibit a high specificity for the interstitial collagen types I, II and III, but little catalytic activity for denatured collagen (gelatin), whereas gelatinases degrade all types of gelatin and show no activity against the interstitial collagens. However, in recent years it has been shown that gelatinases degrade collagen types IV, V, VII and XI (2–9). Collagenase and stromelysin as well as gelatinase are synthesized as latent enzymes. While the activation process in vivo is not fully understood, in vitro activation is achieved by the action of organomercurial compounds such as mersalylic acid or 4-aminophenylmercuric acetate.

Essentially two forms of gelatinases have been described so far: gelatinases secreted from connective tissue cells (fibroblasts) predominantly show molecular masses of about 70 000, while circulating neutrophils, monocytes and macrophages secrete gelatinases of molecular masses 90 000–97 000 (2–4, 10–14). However, the molecular mass of a gelatinase isolated from human skin was 120 000–150 000, as determined by gel filtration and about 100 000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (15).

In this study, we describe a gelatinase of human rheumatoid synovial fluid cells. While rheumatoid synovial cells derived from synovial tissue secrete a connective tissue gelatinase (16), the adherent cells, derived from synovial fluid, released a macrophage-like gelatinase.

Materials and Methods

Materials

Rheumatoid synovia originating from patients of the Rheuma-Klinik, Immanuel-Krankenhaus, Berlin. Buffy coat was kindly supplied by Deutsches Rotes Kreuz, Berlin. Ultrogel AcA 44 and phorbol myristate acetate were purchased from Serva, Heidelberg (Germany). Gelatin-Sepharose was prepared in our laboratory. Sepharose 4-B was obtained from Pharmacia. Cyanogen bromide, gelatin, mersalylic acid, 4-aminophenylmercury acetate, goat anti-rabbit IgG peroxidase conjugate, 3-amino-9-ethylcarbazole and molecular weight markers for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate were purchased from Sigma. The dinitrophenyl-peptide was obtained from Bachem, Heidelberg (Germany). Aprotinin was a gift of Dr. Herbert Wenzel, Bielefeld (Germany).

Protein determination

Protein concentrations were determined with the bichinchoninic acid reagent (Pierce, USA) with bovine albumin as standard.

SDS-PAGE

SDS polyacrylamide gel electrophoresis was performed essentially as described (17).

Immunoblotting

For electroblotting the samples were transferred from the gel to Millipore Immobilon-membranes at 500 mA for 30 min. The membranes were blocked, incubated with antiserum raised in rabbits against leukocyte gelatinase, followed by peroxidase-labelled anti-IgG. The reactive bands were visualized with 3-amino-9-ethylcarbazole.

Enzyme assays

Gelatinase activity was determined with the synthetic substrate N-(2,4)-dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-*D*Arg (18). The activity against protein substrates, such as gelatin and casein, was determined with the fluorescamine method (19).

Enzyme activation

To activate latent gelatinase with organomercurial compounds, mersalylic acid or 4-aminophenylmercury acetate were added to the incubation mixture to give a final concentration of 1 and 1.5 mmol/l respectively. Due to its own fluorescence, 4-aminophenylmercury acetate is not suitable for the fluorescamine method. For the activation with trypsin, the latent enzyme was preincubated for 30 min with 10 µg trypsin. Finally, trypsin was inhibited by the addition of 10 µg aprotinin.

Cell preparation and culture

About 6 ml rheumatoid synovial fluid in a culture flask (25 cm^2) were allowed to stand at 37 °C for 24 hours in an atmosphere of 0.05 CO_2 and 0.95 air. The synovial fluid was decanted from adherent cells and replaced by *Dulbecco*'s modified *Eagle*'s medium containing 100 · 10^3 U/l penicillin, 100 mg/l streptomycin, 1–2.5 mg/l amphotericin B and 0.3 g/l *L*-glutamine without serum. The medium was changed daily, and harvests of culture medium were pooled continuously from the second day until the gelatinase activity was 50 mU/l. The pooled medium was stored at −20 °C.

The determination of cell types was kindly carried out by Professor *Merker*, Institute for Anatomy, Freie Universität Berlin. For this purpose 1 ml of rheumatoid synovial fluid was cultivated as described above on glass coverslips (5 cm^2). These coverslips were fixed in 20 g/l formaldehyde on day 1, 2, 3, 4, 5, 6 and 7 after the start of cultivation and stained with 10 g/l *Giemsa*-solution.

The following steps were carried out at 4 °C.

Chromatography on Ultrogel AcA 44

The crude gelatinase extract was concentrated by ultrafiltration (Amicon, YM30) and subjected to gel chromatography on an AcA 44 column (2 × 90 cm), equilibrated in buffer A (0.05 mol/l Tris-HCl, pH 7.0, 0.2 mol/l NaCl, 0.005 mol/l $CaCl_2$, 0.5 g/l NaN_3, 0.5 g/l Brij 35).

Chromatography on gelatin-Sepharose

The gelatinase-containing fractions from AcA 44 were chromatographed on a gelatin-Sepharose column (1 × 5 cm) equilibrated in buffer A. Elution of gelatinase was achieved with 0.05 mol/l Tris-HCl, pH 7.0, 1 mol/l NaCl, 0.005 mol/l $CaCl_2$, 0.5 g/l NaN_3, 0.5 g/l Brij 35 containing 50 g/l dimethyl sulphoxide (4).

Preparation of leukocytes

The leukocytes were prepared from buffy coat (1000 ml, 4 × 10^{10}/l leukocytes) essentially as described (20).

Phorbol-myristate-acetate stimulation

The leukocytes were stimulated with phorbol myristate acetate essentially as described (4). The leukocyte sediment was suspended in 500 ml *Hank*'s medium, containing Ca^{2+} and Mg^{2+}, at 37 °C. Exocytosis of latent gelatinase was induced by adding 10 µl phorbol myristate acetate (5 mg phorbol myristate acetate in 1 ml dimethyl sulphoxide) and gentle shaking for 20 min. The cells were sedimented by centrifugation at 10 000 *g* for 5 min and the supernatant, containing the gelatinase, was concentrated by ultrafiltration (Amicon, YM30).

Results

Proteinase activity in cell culture medium

Cell adhesion was achieved at more than 80% under the conditions employed (49 cases). Figure 1 shows the synovial cell monolayer culture at various times of cultivation. The adherent cells derived from rheumatoid synovial fluid on day 1 consisted predominantly of macrophages (60%) and fibroblasts (20%) and about 20% of partly degenerating granulocytes. The proportion of fibroblasts increases continuously from about 20% (day 2) to about 70% (day 7), whereas the macrophages decreased from approximately 80% on day 2 to 30% on day 7. After the second day granulocytes were no longer observed.

In the rheumatoid synovial fluids as well as in the media of the adherent synovial cells we found a metal-dependent proteinase activity against the dinitrophenylpeptide. In the synovial fluid and in the initial me-

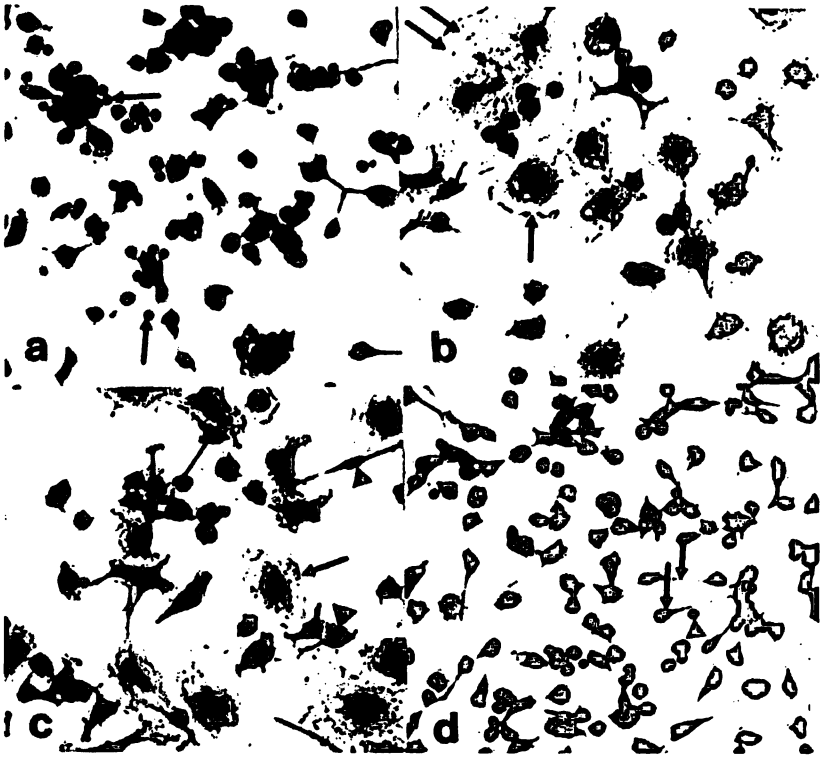

Fig. 1. Photomicrographs (× 200) of synovial fluid cell-monolayer;
a) after 1 day of cultivation: fibroblasts, macrophages and small, round, partly degenerated granulocytes (marked by arrows);
b) after 3 days of cultivation: fibroblasts and many macrophages (marked by an arrow). A giant macrophage is marked by a double arrow;
c) after 5 days of cultivation: fibroblasts (marked by an arrow head) and macrophages (marked by an arrow);
d) after 7 days of cultivation: predominantly fibroblasts (marked by an arrow). Small nuclei (arrow head) indicate the beginning of cell necrosis.

dium (24 hours in culture) this activity was not necessarily latent, but often predominantly in an active form (data not shown), and could be attributed to several proteinases, whereas the activity in the later media (48 hours in culture) was at least 90% latent in all cases. From the third day onwards, no other activity than the latent activity could be detected. Chromatography on Ultrogel AcA 44 of several individual media showed that a single proteinase was responsible for the latent activity.

The gelatinolytic activity found in the media of various cell cultures differed considerably. In a few cases the activity decreased rapidly within two or three days to below 50 mU/l. More typically, the activity, which differed considerably between cultures, decreased more slowly (fig. 2).

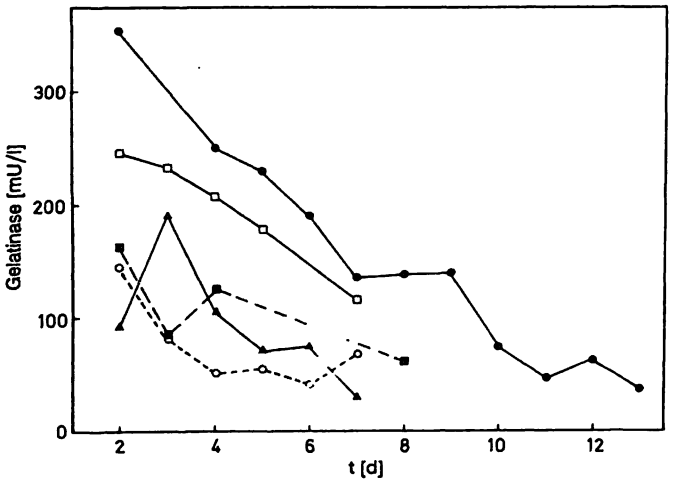

Fig. 2. Time course of dinitrophenyl-peptide hydrolysis by a gelatinase in five different culture media of rheumatoid synovial fluid cells. The media were changed daily.

Gelatinase isolation

The gelatinase from the culture medium conditioned by the human rheumatoid synovial cells was purified by gel chromatography on Ultrogel AcA 44, followed by affinity chromatography on gelatin-Sepharose (figs. 3 and 4). The steps of purification are illustrated (tab. 1). For comparison, we purified the gelatinase from phorbol ester-stimulated human polymorphonuclear leukocytes essentially in the same way. Both gelatinases were eluted from the AcA 44 column with a relative molecular mass of 130 000 – 150 000. The

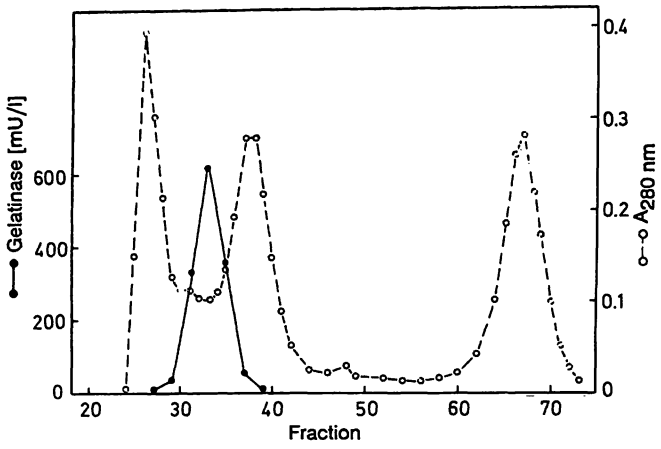

Fig. 3. Gel filtration on Ultrogel AcA 44. Culture supernatants (57 ml), concentrated 10 fold by pressure dialysis, were applied to an Ultrogel AcA 44 column (2 × 90 cm), equilibrated in 0.05 mol/l Tris-HCl, pH 7.0, 0.2 mol/l NaCl, 0.005 mol/l $CaCl_2$, 0.5 g/l Brij 35, 0.5 g/l NaN_3. Fraction volume 4.8 ml.

specific activity of the polymorphonuclear leukocyte gelatinase is about five times higher than the gelatinase isolated from synovial fluid cells, probably due to the higher purity of the enzyme preparation from leukocytes.

SDS-PAGE and immunoblotting

The purified gelatinases from rheumatoid synovial fluid cells and from human neutrophil granulocytes, respectively, showed the same relative molecular mass of about 95 000 in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Antibodies raised against the granulocyte enzyme also recognized the synovial gelatinase (fig. 4).

Substrate specificity and enzyme activation

The gelatinase from synovial fluid cells and from human neutrophil granulocytes displayed an identical behaviour against protein substrates: casein was a much poorer substrate than gelatin and bovine serum albumin was not hydrolysed by either enzyme (fig. 5). Both gelatinases were most effectively activated by trypsin, followed by 4-aminophenylmercury acetate. Activation with mersalylic acid was less efficient (fig. 6).

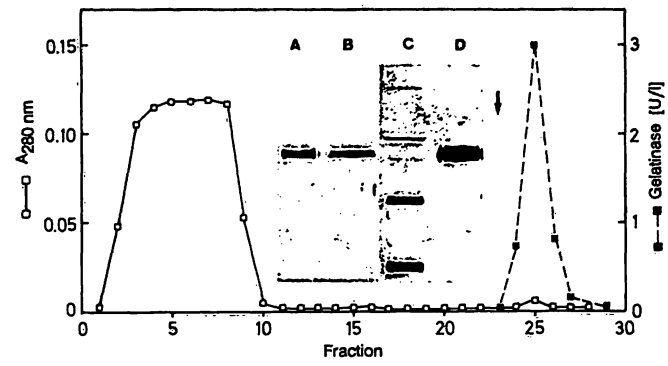

Fig. 4. Gelatin-Sepharose chromatography. The pool from Ultrogel AcA 44 containing gelatinase activity was applied to a column of gelatin-Sepharose, equilibrated in 0.05 mol/l Tris-HCl, pH 7.0, 0.2 mol/l NaCl, 0.005 mol/l $CaCl_2$, 0.5 g/l Brij 35, 0.5 g/l NaN_3. Bound protein was eluted with 0.05 mol/l Tris-HCl, pH 7.0, 1.0 mol/l NaCl, 0.005 mol/l $CaCl_2$, 0.5 g/l Brij 35, 0.5 g/l NaN_3, containing 50 ml/l dimethyl sulphoxide (arrow); fraction volume 4.8 ml. Insert: 8% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and an immunoblot of synovia and polymorphonuclear leukocyte gelatinase.
Lane A 500 ng polymorphonuclear leukocyte gelatinase;
Lane B 2.5 µg synovia gelatinase;
Lane C molecular weight markers (M_r): myosin (205 000), β-galactosidase (116 000), phosphorylase b (97 400), bovine albumin (66 000) and ovalbumin (45 000);
Lane D 10 µg synovia gelatinase.

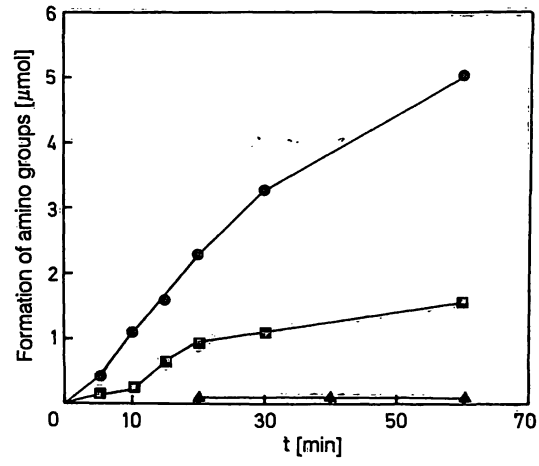

Fig. 5. Degradation of protein substrates. Equal concentrations of the substrates were incubated with gelatinase (enzyme : substrate ratio 1 : 100). Samples were removed at specified times and formation of amino groups was quantified by reaction with fluorescamine. Gelatin (solid circles), casein (solid squares) and bovine albumin (solid triangles).

Tab. 1. Purification of a M_r = 92 000 gelatinase from the culture medium of rheumatoid synovial fluid cells.

Step	Protein (mg)	Activity (mU)	Recovery (%)	Specific activity (mU/mg)	Purification factor
1. Conc. medium	84.0	17.0	100	0.2	(1)
2. Ultrogel AcA 44	3.9	15.3	90	3.9	19.6
3. Gelatin-Sepharose	0.036	12.0	71	333.0	1670.0

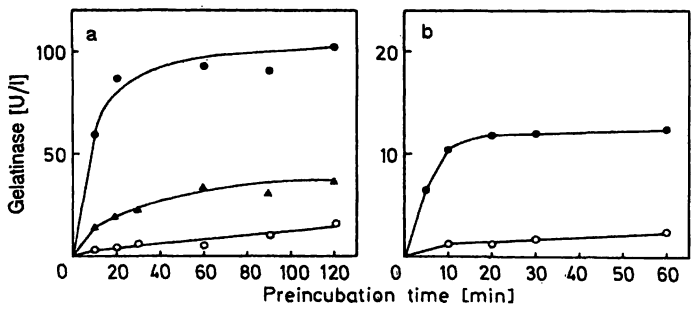

Fig. 6. Activation of latent gelatinase. Progelatinase from human synovial fluid cells was preincubated at 37 °C in the presence of 10 µg trypsin, 0.0015 mol/l 4-aminophenylmercury acetate and 0.001 mol/l mersalylic acid, respectively, for varying periods of time. Before starting the reaction trypsin activity was stopped with 10 µg aprotinin.
Substrates were
dinitrophenyl-peptide (a) and gelatin (b);
activators were:
trypsin (solid circles), 4-aminophenylmercury acetate (solid triangles) and mersalylic acid (open circles).
An identical pattern is obtained with latent gelatinase from human polymorphonuclear leukocytes.

Discussion

In the present study, a fully latent gelatinase was isolated from the culture medium of human rheumatoid synovial fluid cells. This enzyme had already been found in the synovia, together with other metalloproteinases hydrolysing the dinitrophenyl-peptide. However, after two or not later than three days in culture, all the proteolytic activity against gelatin or the dinitrophenyl-peptide, respectively, was due to the latent gelatinase, which could be detected for about two weeks in the culture.

The gelatinase had a relative molecular mass of 130 000 – 150 000 on Ultrogel AcA 44 and displayed a molecular mass of about 95 000 in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. These data are in full agreement with those for human leukocyte gelatinase. Both enzymes showed identical behaviour with respect to substrate specificity and activation with trypsin or organomercurial compounds, such as 4-aminophenylmercury acetate and mersalylic acid. In contrast to other reports (1, 7), our data clearly indicate that trypsin is much more efficient than the organomercurials 4-aminophenylmercury acetate and mersalylic acid. The gelatinase from synovial cells reacted with antibodies raised against human granulocyte gelatinase. Thus, the gelatinase we isolated from human rheumatoid synovial fluid cells is clearly a leukocyte-type of gelatinase.

Polymorphonuclear leukocytes, which can amount to more than 70% of all leukocytes in rheumatoid synovial fluid (21), can be excluded as the source of this gelatinase, because under the conditions employed, polymorphonuclear leukocytes do not survive longer than maximally 24 – 48 hours (22, 23). In accordance with the literature, we found 20% polymorphonuclear leukocytes after one day of culture. On the second day this cell type could not longer be detected in our monolayer cultures. For a more detailed discussion of the cell types found in cultures of synovial fluid cells see l. c. (24).

Human monocytes/macrophages have been reported to secrete a latent metalloproteinase with a relative molecular mass of 92 000. Thus it can be concluded that the gelatinase described in this communication is secreted by monocytes/macrophages and/or macrophage-like synovial A cells.

Without stimulation, freshly isolated human monocytes from non-rheumatoid patients did not synthesize detectable amounts of this proteinase (25, 27). However in later culture media, when the monocytes had transformed to macrophages (10 – 14th day in the culture), a gelatinase activity could be detected (26). A similar secretion behaviour can be expected from the macrophage-like A synovial cells, which are most probably derived from blood monocytes (28). At the start of the cell culture we found gelatin-degrading activity in the culture media and in the rheumatoid synovial fluid. If this activity had been contributed by blood monocytes, which constituted 80% of the cells in the early culture medium, these monocytes should have been in a stimulated state, possibly due to some factors present in the rheumatoid synovial fluid.

Fibroblasts, which are the predominant cell type in late cultures, produce a different type of gelatinase with a relative molecular mass of 72 000. Transformed human lung fibroblasts secrete the M_r = 72 000 gelatinase as well as the M_r = 92 000 gelatinase (8). However, in one case, it was reported that human gingiva fibroblasts secreted the typical fibroblast M_r = 72 000 gelatinase with minor quantities of the M_r = 92 000 gelatinase (25). The authors reported that the production of the M_r = 92 000 gelatinase is not unusual in fibroblasts of other species than human.

Considering these various possibilities it seems difficult to determine exactly the cellular origin of the gelatinase in our cultures. However, we believe, in accordance with the observed time-dependent decrease of macrophages/monocytes in our cultures, that the gelatinase is most probably released by either the macrophage-like synovial A-cells or the activated blood monocytes. Fibroblasts are stimulated by macrophages to secrete the M_r = 72 000 gelatinase (16).

But in our system only the M_r = 92000 gelatinase could be detected, which is a surprising and in our opinion important observation in rheumatoid synovial cells, because both enzymes obey different regulatory mechanisms. The degree of tissue degradation depends ultimately on the ratio of active proteinases to proteinase inhibitors. Because the metalloproteinases, collagenase, stromelysin and gelatinase, are synthesized as inactive precursors, their activation is an important step in the regulated turnover of the extracellular matrix. With respect to activation and inhibition, there are marked differences between fibroblast and leukocyte gelatinase. Serine proteinases, such as trypsin, cathepsin G and elastase, are potent activators of leukocyte gelatinase (12, 29–32). In contrast, the fibroblast gelatinase is not activated by these serine proteinases (5, 33). Both enzymes (leukocyte and fibroblast gelatinase) also show different behaviour against the tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2). While TIMP-1 exclusively binds to the latent leukocyte gelatinase, TIMP-2 exclusively binds to the latent fibroblast gelatinase in a 1 : 1 stoichiometry (8, 34–36). The activated enzymes are inhibited by both TIMPs. However, TIMP-1 is reported to have a more limited inhibitory potency against activated polymorphonuclear leukocyte metalloproteinases (3, 37, 38).

Fibroblast gelatinase plays a physiological role in remodelling of the extracellular matrix. The M_r = 92000 gelatinase has the same capacity in degrading the components of the extracellular matrix. As this enzyme, however, is subjected to different control mechanisms, its secretion by rheumatoid synovial cells may be one of the factors responsible for the destructive processes in rheumatoid arthritis.

Acknowledgement

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